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A [3]Ferrocenophane Polyphenol Showing a Remarkable Antiproliferative Activity on Breast and Prostate Cancer Cell Lines

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^a ER, estrogen receptor; RBA, relative binding affinity; CV, cyclic voltammogram; QM, quinone methide; CD, partially methylated β -cyclodextrin; Fc, ferrocene.

We have previously shown that modification of polyphenols with a ferrocenyl group can dramatically enhance their cytotoxicity. We now present two new [3]ferrocenophane compounds, one of which has an antiproliferative effect seven times stronger than the corresponding noncyclic species, with IC₅₀ values of 90 and 94 nM on hormone-independent MDA-MB-231 breast and PC-3 prostate cancer cell lines, respectively. Solubility studies in water using methylated β -cyclodextrin and electron transfer studies are also presented.

Introduction

Bioorganometallic chemistry is driving an increasing amount of growth in drug design.¹⁻³ As part of an ongoing study of biologically active organometallic compounds, we have described a reasoned approach to preparing a new family of metallocene complexes with strong antitumor potential. This approach involves the substantial modification of the biological properties of polyphenols by the covalent addition of a ferrocenyl group to the organic skeleton.⁴⁻¹¹ Compound **1** (Chart 1) is estrogenic on MCF-7 hormone-dependent breast cancer cells, promoting cell proliferation, while **2**, bearing a ferrocenyl entity, produces a powerful antiproliferative effect on both MCF-7 cells and MDA-MB-231 hormone-independent breast cancer cells.⁴ Likewise, while the natural polyphenol resveratrol **3** has an IC₅₀ value as high as 20 μ M on MDA-MB-231 cells,¹² the IC₅₀ value for **2** is on the order of 0.5 μ M,⁴ a 40-fold improvement. Thus, although organic polyphenols are not efficacious at a therapeutic level, modification with Fc^a vastly improves their antiproliferative properties in vitro.

It should be emphasized that the simple presence of a ferrocenyl group is not sufficient to generate cytotoxic compounds.¹¹ For example, a proliferative effect was found on estrogen receptor (ER) positive cells for the molecule created by the attachment of a ferrocenyl group to the 17 β position of the natural hormone estradiol.¹³ Both the position and the structural pattern in which Fc is inserted are important, and the motif [Fc]-[conjugated spacer]-[*p*-phenol] seems to be crucial for strong cytotoxic effects; a change in the position of either the OH or ferrocenyl group resulted in a weakened cytotoxic effect.⁵⁻⁹

Our aim is now to seek related molecules that may have even higher efficacy than **2**. We envisaged solutions based on new structures while preserving the key ferrocenyl-phenol motif, and it seemed to us that constrained ring structures were particularly appropriate. Rigid molecules can bind more strongly to a receptor than their flexible analogues if they have the proper geometry for entering the active site, while a flexible molecule must adopt such a geometry, causing entropy loss and weaker binding. Accordingly, molecules **4** and **5**, based on the [3]ferrocenophane motif, were prepared and studied (Chart 2). Molecule **4** possesses a direct linkage between the cyclopentadienyl ring

and the double bond, while the other cyclopentadienyl is attached to the double bond by a two-carbon bridge. In **5**, the ferrocenyl is symmetrically linked to the double bond by a single-carbon bridge.

Results

Compound **4** was obtained in 28% yield via a McMurry cross-coupling reaction with the ferrocenophanone **6** and 4,4'-dihydroxybenzophenone (Scheme 1). Compound **6** could be obtained via the reaction of Fc with acryloyl chloride in the presence of aluminum chloride,^{14,15} or alternatively, via the acylation of Fc with ethyl *mono*-malonate,¹⁶ followed by Clemmensen reduction of the resulting ketoester and hydrolysis and cyclization of the ferrocenylpropionic acid formed. The synthesis of **5** required 1,1'-(2-ketotrimethylene)ferrocene, which has been previously reported.¹⁷ A McMurry cross-coupling reaction of this compound with 4,4'-dihydroxybenzophenone gave **5** as a yellow powder in 22% yield.

The relative binding affinity (RBA) to ER, lipophilicity, and proliferative/antiproliferative effects against the MCF-7 ER+ breast, the MDA-MB-231 ER- breast, and the PC-3 AR- prostate cancer cell lines are reported in Table 1, along with the values obtained for the noncyclic compound **2** for comparison. Molecules **4** and **5** recognize ER α and ER β , present in varying degrees in MCF-7 cells, with overall values on the same order of magnitude as that of **2**. However, **4** and **5** exhibit different behavior on the growth of MCF-7 cells. After five days of culture, **4** is estrogenic at low concentration (10 nM; 124% vs control) and becomes cytotoxic at higher concentration (100 nM; 69%). At 10 nM, **5** is estrogenic after 4 days of culture (118%) but, unusually, becomes cytotoxic after 5 days of culture (80%). IC₅₀ values of 4 μ M for **4** and of 1 μ M for **5** can be estimated for MCF-7 cells after five days of incubation. It is clear that there is a competition on this cell line between the estrogenic (positive) and cytotoxic (negative) effects, and it seems from these results that **4** is more estrogenic than **5**. Surprisingly, this could not be predicted from the RBA values, which are quite similar. Overall, the values for MCF-7 cells are on the same order of magnitude as that of the noncyclic diphenol **2**.

Compound **4** shows an exceptional antiproliferative effect on the hormone-independent

MDA-MB-231 and PC-3 cells, with an IC_{50} value of $0.09 \mu\text{M}$, an order of magnitude more cytotoxic than **5**, which lacks conjugation between the phenol and the ferrocenyl groups. The cytotoxic effect of compound **2**, lacking the ferrocenophane structure but possessing a conjugated system, falls intermediate between **4** and **5**.

Table 1. RBA, $\log P_{O/w}$, and the Effect on Cancer Cell Growth of **2**, **4**, and **5**

	RBA(%)		$\log P_{O/w}$	$IC_{50} (\mu\text{M})^a$	
	ER α	ER β		MDA-MB-231	PC-3
2	9.6 ± 0.9	16.3 ± 1.5	5.0	0.64 ± 0.06	0.7 (1 exp)
4	7.2 ± 0.7	4.84 ± 0.4	4.6	0.09 ± 0.01	0.094 ± 0.006
5	7.6 ± 0.6	15.4 ± 0.4	4.8	0.96 ± 0.03	1.08 ± 0.02

^a Measured after 5 days of culture (mean of two independent experiments).

Chart 1. Replacement of Ph with Fc on **1** Imparts Cytotoxicity

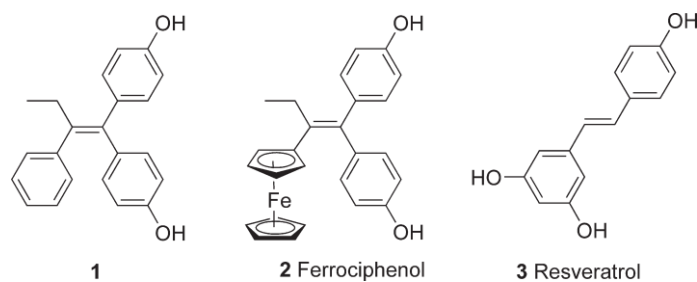
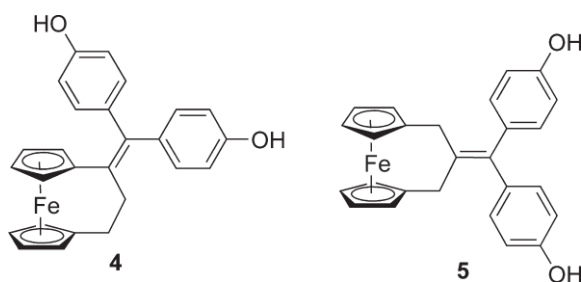
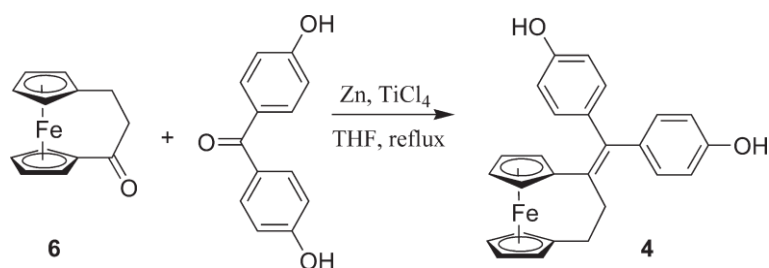


Chart 2. Two New Cytotoxic [3]Ferrocenophanes Studied in This Report



Scheme 1



The presence of partially methylated β -cyclodextrin (CD) does not hinder the in vitro cytotoxic effects of **4**. MDA-MB-231 incubation with the CD-**4** complex (obtained by addition to an aqueous solution of 2 equiv of CD and allowed to dissolve overnight) gave identical results between the free and encapsulated forms (10% cell survival at 10^{-6} M). The complexation of **4** with CD in water was investigated by electrochemistry, as previously reported for **2**.¹⁸ Qualitatively, the time required to dissolve **4** in H₂O in the presence of CD was fast compared to that of **2** (20 min vs 1 h). The cyclic voltammogram (CV) of the Fc/Fc⁺ redox couple obtained with the solution of CD-**2** (see Supporting Information) exhibited a plateau shape characteristic of a consecutive chemical-electrochemical mechanism, indicating that the oxidation process was limited by the dissociation step of the complex formed between the CD and compound **2**. The peak shape and the faster electron transfer obtained with CD-**4** are thus consistent with a weak complexation and a fast decomplexation dynamic.

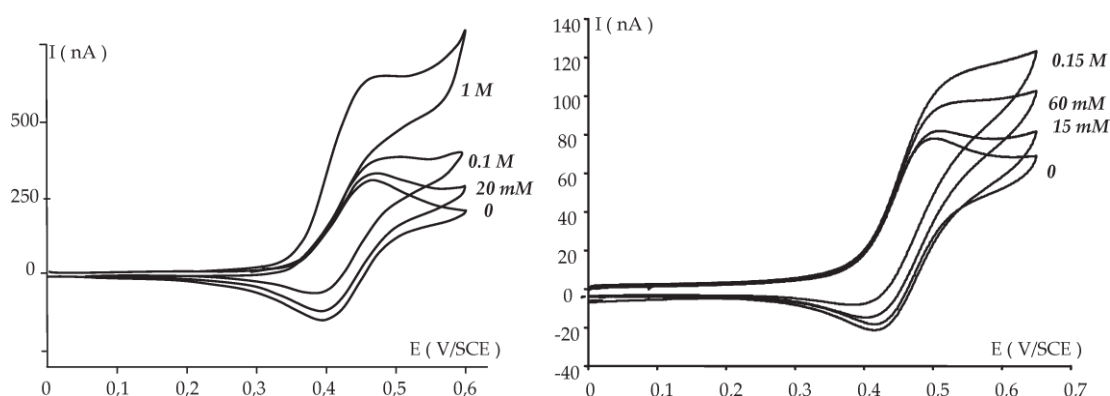


Figure 1. Cyclic voltammograms of **4** (1 mM, scan rate 100 mV/s, left) and **5** (0.3 mM, scan rate 50 mV/s, right) in the presence of varying concentrations of imidazole.

As observed in Figure 1, in the absence of imidazole, compounds **4** and **5** exhibit a monoelectronic oxidation process at 0.46 V (**4**) and 0.48 V (**5**), ascribed to the ferrocenyl oxidation. Upon addition of increasing amounts of base, the intensities of the oxidation waves of both compounds increase, indicating an electron transfer between the phenol and the ferricenium groups prior to the reverse sweep.

Discussion and Conclusions

Compound **4** represents a major advance in the study of antiproliferative organometallic compounds, with an IC₅₀ value on ER- cells several times lower than our noncyclic lead compound **2**. On ER+ cells, **4** displays a combination of estrogenicity and cytotoxicity, which suggests that it should be more efficacious against hormone-refractory tumors. However, to create a compound active against both types of cells, one could eliminate the estrogenic effect by replacing one hydroxyl group with the lateral chain -O(CH₂)_xN(CH₃)₂ (*x*=3-5), as we have previously shown for the hydroxyferrocifen series.¹⁹

Compound **4** is itself too hydrophobic to envision its intravenous administration, thus possibly limiting its bio-availability for clinical applications. The potency of **4** against MDA-MB-231 cells when encapsulated by partially methylated β -cyclodextrin was maintained. The ability of cyclodextrins to include part of an organometallic complex in their internal hydrophobic cavities has been amply demonstrated,²⁰ and both the stoichiometry and the orientation of ferrocenyl depend on the size of the cyclodextrin. The fast complexation/decomplexation dynamic observed suggests that the ferrocenyl group is situated in an equatorial orientation²¹ in such a way that hydrophobic interactions between the phenyl and methoxy groups are minimized.

One of the possible mechanisms that we have identified to explain the cytotoxic effect of ferrocenyl phenols on various cancer cells is based on the in situ transformation to a quinone methide (QM), a process that is mediated by the ferrocenyl group and can be followed by electrochemistry.⁹ Electrochemical experiments suggest that transformation to a QM can be realized for **4** and **5**. In this context, the intramolecular proton coupled electron transfer from the phenol to the ferricenium in **4** can be explained by the classical π -delocalized mechanism. For the unconjugated **5**, one must consider either that the

electron transfer proceeds “through space”, or via the formation of an intermediate α -methylene radical (such α -methylene ferricenium molecules possess acidic protons),²² which can delocalize over the π system and undergo an additional oxidation step to yield the QM.

It has recently been shown that resveratrol can affect cancer cell growth and induce apoptosis in launching the synthesis of endogenous ceramide, a bioactive sphingolipid.^{12,25} Ceramide is a promising pharmacological target, particularly for cancer therapy. Many drugs that promote the production of ceramide have been proven to be more effective on malignant cells than on healthy tissue,¹² and this is also the case with compound **2**.²³⁻²⁵ The idea that compound **4**, the most effective to date, could possess a high ceramide-mediated proapoptotic activity on human breast cancer cells is however just a working hypothesis and will be explored in the future. It remains that **4** shows a very strong antiproliferative effect against hormone independent cancer cell lines, and this next generation of cyclic ferrocenyl phenols is currently being expanded for future study.

Experimental Section

All reactions were performed under inert atmosphere, THF was distilled from Na/benzophenone, and commercial reagents were used without further purification. Purity of >95% was confirmed via elemental analysis for **4** and analytical HPLC for **5**.

1,1'-{1-[1,1-Bis(4-hydroxyphenyl)methylidene]trimethylene} ferrocene (4). To a suspension of 3.07 g (46.9 mmol) of Zn dust in 100 mL of THF and 4.55 g (2.64 mL, 24 mmol) of TiCl₄ was added at -10 °C. The cooling bath was removed, and the mixture was refluxed for 1 h. After cooling to rt, 3.79 g (3.87 mL, 47.9 mmol) of anhydrous pyridine was added and the mixture was stirred for 5 min. A solution of 1.92 g (8 mmol) of [3]ferrocenophan-1-one and 1.71 g (8 mmol) of 4,4'-dihydroxybenzophenone in 30 mL of THF was added and the mixture was refluxed for 90 min. After cooling to rt, the mixture was hydrolyzed with 100 mL of 8% aq K₂CO₃. The reaction mixture was extracted with several 100 mL portions of diethyl ether. The organic phase was washed with water (2x200 mL) and brine (200 mL), dried over MgSO₄, and evaporated to dryness. Product **4** (2nd yellow fraction) was isolated by flash

chromatography on silica gel (*n*-pentane:diethyl ether 2:1). Yield=1.57 g (47%, purity 90-95%). A second column using 60 mL of silica gel and *n*-pentane:diethyl ether 4:1 yielded a yellow powder, which was recrystallized from acetone or from a mixture of ethyl acetate (5 mL) and *n*-pentane (80 mL) at 4 °C. After 48 h, the organic solution was removed and the yellow crystals washed twice with *n*-pentane. Yield=0.95 g (28%). ¹H NMR (300.13 MHz, acetone-*d*₆): 8.31 (s, 1H, OH), 8.10 (s, 1H, OH), 7.07 (d, *J*=8,7 Hz, 2H, Ar), 6.85 (d, *J*=8,7 Hz, 2H, Ar), 6.84 (d, *J*=8,7 Hz, 2H, Ar), 6.54 (d, *J*=8,7 Hz, 2H, Ar), 4.25 (t, *J*=1,7 Hz, 2H, C₅H₄), 3.98 (m, 4H, C₅H₄), 3.92 (t, *J*=1,7 Hz, 2H, C₅H₄), 2.70 (m, 2H, CH₂), 2.34 (m, 2H, CH₂). ¹³C NMR (75.48 MHz, acetone-*d*₆): 157.0 (C), 156.4 (C), 141.5 (C), 136.0 (C), 135.9 (C), 133.4 (C), 132.4 (2CH), 131.2 (2CH), 115.7 (2CH), 114.8 (2CH), 87.6 (C C₅H₄), 84.9 (C C₅H₄); 71.0 (2CH C₅H₄), 70.8 (2CH C₅H₄), 69.1 (2CH C₅H₄), 68.8 (2CH C₅H₄), 41.5 (CH₂), 29.1 (CH₂). Analysis: calcd for C₂₆H₂₂FeO₂: C 73.95%, H 5.25%; found: C 73.79%, H 5.34%. HRMS (CI — CH₄): calcd for C₂₆H₂₂FeO₂: 423.1047; found: 423.1040 (M+H)⁺.

1,1’-{2-[1,1-Bis (4-hydroxyphenyl) methylidene] trimethylene} ferrocene (5). Compound **5** was synthesized as described for **4** but at 5% scale, with similar workup. Yield=0.038 g (0.090 mmol, 22%) of yellow powder. ¹H NMR (300.13 MHz, acetone-*d*₆): 8.22 (s, 2H, OH), 7.16 (d, *J*=8.7 Hz, 4H, Ar): 6.80 (d, *J*=8.7 Hz, 4H, Ar), 4.11 (t, *J*=1.9 Hz, 4H C₅H₄), 3.99 (t, *J*=1.9 Hz, 4H, C₅H₄), 2.81 (s, 4H, CH₂). ¹³C NMR (75.48 MHz, acetone-*d*₆): 156.7 (C), 141.7 (C), 138.6 (C), 135.8 (C), 131.0 (2 x 2CH), 115.7 (2 x 2 CH), 83.5 (2 C C₅H₄), 70.3 (2x 2CH C₅H₄), 66.9 (2x2CH C₅H₄), 28,3 (2CH₂). IR (KBr, cm⁻¹): 1609, 1508, 1430, 1220, 1197, 831. HRMS (CI-CH₄): calcd for C₂₆H₂₃FeO₂: 423.1047 (M+H)⁺; found: 423.1034 (M+H)⁺.

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Supporting Information Available: Analytical instrumentation, modified synthesis of **4**, biochemical testing conditions, and CV of CD-2 and CD-4. This material is available free of charge via the Internet at <http://pubs.acs.org>. (or see the end of this file)

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Supporting Information

Analytical Instrumentation

Infrared spectra were obtained on an IRFT BOMEM Michelson-100 spectrometer equipped with a DTGS detector as a KBr plate. ^1H and ^{13}C NMR spectra were recorded on a 300 MHz Bruker spectrometer. Mass spectrometry was performed with a Nermag R 10-10C spectrometer. Melting points were measured with a Kofler device. Elemental analyses were performed by the microanalysis service of CNRS at Gif sur Yvette. Electrochemical experiments were performed. Cyclic voltammograms were obtained utilizing a Princeton Applied Research potentiostat. Analyte solutions were 1 mM in DMF with 0.1 M Bu_4NBF_4 supporting electrolyte. Cyclic voltammetry experiments were performed at room temperature under an argon atmosphere in a three-electrode cell. The reference electrode was an SCE (Tacussel), which was separated from the solution by a bridge compartment filled with the same solvent/supporting electrolyte solution as used in the cell. The counter electrode was a platinum mesh (Goodfellow). The platinum working electrode was home-made (0.5 mm diameter; Goodfellow).

Synthesis of Intermediates

Ethyl ferrocenoylacetate, 6 was prepared by slightly modified procedure for acylation of ferrocene with acyl trifluoroacetate.¹ To a stirred solution of 13.2 g of ethyl *mono*-malonate and 21 g (14 ml) of trifluoroacetic anhydride in 100 ml of dichloromethane was added 18.6 g of ferrocene. After 5 min a solution of 30 ml of methanesulfonic acid in 50 ml of dichloromethane was added. The resulting violet mixture was stirred at room temperature. After 4 h the reaction mixture was poured onto ice (300g) and the resulting solution was neutralized by addition of solid NaHCO_3 . Products were extracted with DCM. Chromatography on silica gel (DCM – ethyl acetate 95 - 5) gave 18 g of pure product. ^1H , ^{13}C NMR confirmed the structure as reported in the literature.²

Ethyl 3-ferrocenylpropionate, 7 was prepared as previously described³ for the synthesis of ferrocenylbutyric acid. To a mixture of 70 g of zinc dust and 10 g of $\text{Hg}(\text{OAc})_2$ in 150 ml of water, 5 ml of 37% HCl was added and the resulting mixture was stirred for 10 min. The aqueous solution was decanted and the Zn/Hg was washed 4 times with 200 ml portions of water. The mixture of Zn/Hg, the solution of 10 g of $\text{FcCOCH}_2\text{COOEt}$ in 160 ml of toluene, 40 ml of water and 60 ml of 37% HCl was stirred and refluxed for 4 h (only one product on TLC – hexane). After cooling to room temperature the organic solution was separated and the inorganic residue extracted with toluene. The toluene solution was washed with sat. aq. NaHCO_3 and dried with MgSO_4 . Evaporation of the solvents gave the product in quantitative yield. The ^1H and ^{13}C NMR spectra confirmed the structure as reported in the literature.⁴

Ferrocenylpropionic acid, 8. A mixture of 9 g of **7**, 25 ml of EtOH, and 100 ml of 20% aq. solution of NaOH was refluxed. After 3 h the resulted solution was diluted with 200ml of water and extracted with several portion of diethyl ether and the aqueous solution was filtered and acidified with conc. HCl to pH 1. **8** was extracted twice with 100 ml portions of DCM, organic solution was washed twice with 200 ml portions of water and dried. Evaporation of solvent gave 5.3 g of pure **8**. Crystallization from DCM – *n*-pentane gave product as a yellow powder. ^1H , ^{13}C NMR confirmed the structure as reported in the literature.⁵

Biochemical studies

Materials. Stock solutions (1×10^{-3} M) of the compounds to be tested were prepared in DMSO and were kept at 4 °C in the dark; under these conditions they are stable at least two months. Serial dilutions in DMSO were prepared just prior to use. Dulbecco's modified eagle medium (DMEM) with phenol red/Glutamax I, Dulbecco's modified eagle medium (DMEM) without phenol red, Glutamax I and fetal bovine serum (FBS) were purchased from Invitrogen; estradiol from Sigma. PC-3 and MDA-MB-231 cells were from American Type Culture Collection (ATCC, LGC Promochem). Sheep uteri weighing approximately 7 g were obtained from the slaughterhouse at Mantes-la-Jolie, France. They were immediately frozen and kept in liquid nitrogen prior to use.

Determination of the Relative Binding Affinity (RBA) of the compounds for ER α and ER β . RBA values were measured on ER α from lamb uterine cytosol and on ER β Panvera purchased from Invitrogen (France). Sheep uterine cytosol prepared in buffer A (0.05 M Tris-HCL, 0.25 M sucrose, 0.1% β mercaptoethanol, pH 7.4 at 25 °C) was used as a source of ER α . For ER β , 10 μ l of the solution containing 3500 pmol/ml were added to 16 ml of buffer B (10% glycerol, 50 mM Bis-Tris-Propane pH = 9, 400 mM KCl, 2 mM DTT, 1 mM EDTA, 0.1% BSA) in a silanized flask. Aliquots (200 μ l) of ER α in glass tubes or ER β in polypropylene tubes were incubated for 3 h at 0 °C with [6,7-3H]-estradiol (2×10^{-9} M, specific activity 1.62 TBq/mmol, NEN Life Science, Boston MA) in the presence of nine concentrations of the hormones to be tested. At the end of the incubation period, the free and bound fractions of the tracer were separated by protamine sulfate precipitation. The percentage reduction in binding of [3H]-estradiol (Y) was calculated using the logit transformation of Y ($\text{logit}Y: \ln[y/1-Y]$) versus the log of the mass of the competing steroid. The concentration of unlabeled steroid required to displace 50% of the bound [3H]-estradiol was calculated for each steroid tested, and the results expressed as RBA. The RBA value of estradiol is by definition equal to 100%.

Culture conditions. Cells were maintained in a monolayer culture in DMEM with phenol red/Glutamax I supplemented with 9% fetal bovine serum at 37 °C in a 5% CO₂/air-humidified incubator. For proliferation assays, PC-3 and MDAMB-231 cells were plated in 1 mL of DMEM without phenol red, supplemented with 9% decompeted and hormone-depleted fetal bovine serum, 0.9% kanamycin, 0.9% Glutamax I and incubated. The following day (D0), 1 mL of the same medium containing the compounds to be tested was added to the plates. After 3 days (D3) the incubation medium was removed and 2 mL of the fresh medium containing the compounds was added. After 5 days the total protein content of the plate was analyzed as follows: cell monolayers were fixed for 1 h at room temperature with methylene blue (1mg mL^{-1} in 50:50 water/MeOH mixture), then washed with water. After addition of HCl (0.1 M, 2 mL), the plate was incubated for 1 h at 37 °C and then the absorbance of each well (6 wells for each concentration) was measured at 655 nm with a Biorad spectrophotometer. The results are expressed as the percentage of proteins versus the control.

Cyclic voltammogram of CD-4

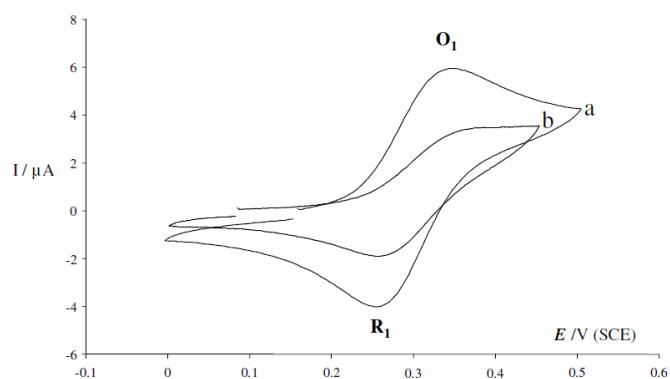


Figure S1. Cyclic voltammogram of a) **4** and b) **2** (1 mM) in H₂O + Na₂SO₄ (0.1 M) recorded at a glassy carbon electrode (3 mm diameter) at 50 mV s⁻¹, in the presence of 20 eq. Me-β-CD. O₁ and R₁ refer to the oxidation and reduction waves, respectively.

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